Matrix vesicles are extracellular organelles involved in mineral formation that are regulated by 1α,25(OH)2D3. Prior studies have shown that protein kinase C (PKC) activity is involved in mediating the effects of 1α,25(OH)2D3 in both matrix vesicles and plasma membranes. Here, we examined the regulation of matrix vesicle PKC by 1α,25(OH)2D3 during biogenesis and after deposition in the matrix. When growth zone costochondral chondrocytes were treated for 9 min with 1α,25(OH)2D3, PKCζ in matrix vesicles was inhibited, while PKCa in plasma membranes was increased. In contrast, after treatment for 12 or 24 h, PKCζ in matrix vesicles was increased, while PKCa in plasma membranes was unchanged. The effect of 1α,25(OH)2D3 was stereospecific and metabolite-specific. Monensin blocked the increase in matrix vesicle PKC after 24 h, suggesting the cells may use local production of the vitamin D metabolite as a mechanism for controlling events in the matrix. A number of observations support this hypothesis. Treatment of matrix vesicles with 1α,25(OH)2D3 causes increased alkaline phosphatase specific activity, which is associated with the onset of calcification. In addition, phospholipase A2 (PLA2) stereospecific activity was increased, which may lead to a loss of membrane integrity and the release of proteases capable of remodeling the matrix. One of the matrix metalloproteinases that are present in matrix vesicles, stromelysin-1 (MMP-3), has been shown to activate latent transforming growth factor β-1 (TGF-β1) in a 1α,25(OH)2D3-dependent manner. The mechanisms involved in the regulation of matrix vesicles by 1α,25(OH)2D3 are not known. 1α,25(OH)2D3 modulates proliferation and differentiation of growth plate chondrocytes via the nuclear vitamin D receptor (1α,25-nVDR) and the concerted action of transcription factors and co-activators. However, matrix vesicles do not contain DNA or RNA, so genomic pathways are unlikely to play a role in mediating the direct effect of 1α,25(OH)2D3 on the organelles.

Recent studies show that 1α,25(OH)2D3 also acts on cells through membrane-mediated mechanisms, resulting in rapid changes in calcium ion flux, phospholipid metabolism and kinase activation, including a rapid increase in protein kinase C (PKC) specific activity. Many of the physiological responses of the chondrocytes to 1α,25(OH)2D3 are blocked by inhibiting PKC. Moreover, both the PKC-dependent responses and the rapid increase in PKC itself are blocked with an antibody generated to a [3H]-1α,25(OH)2D3-binding protein present in the basal lateral membranes of chick intestinal epithelium (Ab99) (15–17). The rapid effects of 1α,25(OH)2D3 are stereospecific; only the 1α,25(OH)2D3 isomer elicits an increase in PKC or regulates the signaling pathways that lead to the increase in PKC (18), indicating a 1α,25(OH)2D3-dependent mechanism.

The hypothesis that 1α,25(OH)2D3 regulates matrix vesicles
via 1,25-mVDR-mediated changes in PKC is attractive. Ab99 recognizes a single protein band in Western blots of matrix vesicles with a $M_\text{s}$ of 65,000, and matrix vesicles exhibit specific binding for [³H]-1,25(OH)₂D₃ (16). Moreover, the effect of 1α,25(OH)₂D₃ on PKC is blocked by Ab99, just as it is in the cell. However, 1α,25(OH)₂D₃ stimulates PKC activity in growth zone chondrocytes and when incubated directly with chondrocyte plasma membranes, whereas it inhibits PKC activity when incubated directly with matrix vesicles (19), even though the same receptor is involved.

The purpose of the present study was to examine the mechanisms that regulate 1α,25(OH)₂D₃-dependent PKC activity in matrix vesicles. There are several reasons why regulation of matrix vesicle PKC might differ from that of the plasma membrane. First, there is a differential distribution of PKC isoforms between the two membrane fractions. PKCζ predominates in matrix vesicles, whereas PKCα predominates in plasma membranes (19). The two membrane fractions differ in other ways as well, including phospholipid composition (20) and basal membrane fluidity (3).

Studies using chondrocytes from the resting zone of costochondral cartilage indicate that the responsive isoform in intact cells, as well as in isolated plasma membranes, is PKCα, whereas the responsive isoform in matrix vesicles is PKCζ. Whether this is also the case for growth zone cells is not known, however. PKC is regulated by 24R,25(OH)₂D₃ in resting zone cells, but 1α,25(OH)₂D₃ regulates activity in growth zone cells, and these two metabolites use two distinctly different mechanisms to regulate PKC activity in their target cells (9, 21). Moreover, matrix vesicle composition, including phospholipids and enzyme activities, and regulation of matrix vesicle function differs between the two cell types (see Refs. 14, 22 for reviews).

It is likely that 1α,25(OH)₂D₃ regulates matrix vesicle PKC during organelle biogenesis, as well as directly, once they are resident in the extracellular matrix. When growth plate chondrocytes are cultured with 1α,25(OH)₂D₃ for 24 h, long enough for new gene expression and matrix vesicle synthesis, matrix vesicle PKC activity is increased (23). While 1α,25(OH)₂D₃ has been shown to regulate the distribution of matrix proteinases in matrix vesicles (24), it is not known if the increase in matrix vesicle PKC is due to preferential incorporation of specific isoforms of the enzyme. PKCα is sensitive to Ca²⁺ ions and to phospholipid, whereas PKCζ is insensitive to both co-factors (25), yet both Ca²⁺ ions and phospholipid are present at relatively high levels in the growth plate extracellular matrix (26), particularly in the growth zone. Thus, it is possible that other isoforms may be involved in the matrix vesicle response to 1α,25(OH)₂D₃. For example, in renal epithelial cells, 1α,25(OH)₂D₃ has been shown to increase PKCβ activity (27). Other aspects of the signaling pathway by which 1α,25(OH)₂D₃ regulates matrix vesicle PKC may differ as well. G-protein, specifically Gαi, but not Gαs, mediates the effect of 1α,25(OH)₂D₃ on cellular PKC (21); whether this is the case for matrix vesicle PKC is unknown.

Phospholipid metabolism plays a major role in the mechanism of 1α,25(OH)₂D₃-dependent PKC activity in the intact cell, causing rapid increases in phospholipase A₂ (PLA₂) and phospholipase C (PLC) activity, release of arachidonic acid and diacylglycerol, and production of prostaglandin E₂ (PGE₂) (9). This may not be the case for matrix vesicles, however. Matrix vesicles possess an active phospholipid metabolism that is regulated independently from that of the cell (28). Their phospholipid composition is distinct from that of the plasma membrane as well (29–31). Unlike the plasma membrane, which has a phospholipid composition higher in phosphatidycholine, matrix vesicles contain higher levels of phosphatidylserine and phosphatidylinositol, as well as cardiolipin. The basal fluidity of the plasma membrane and matrix vesicles also differs (3). Thus, it is likely that phospholipid metabolism may play a different role in the mechanism by which 1α,25(OH)₂D₃ modulates PKC activity in the extracellular organelle.

This study tested the hypothesis that 1α,25(OH)₂D₃ regulates matrix vesicle PKC activity in multiple ways. The vitamin D metabolite first increases the amount of PKCζ incorporated during matrix vesicle biogenesis though genomic mechanisms. Once the matrix vesicles are released into the matrix, 1α,25(OH)₂D₃ acts directly on the matrix vesicle via the 1,25-mVDR, reducing PKCζ activity. The signaling pathways differ from those that participate in the increase in plasma membrane PKC activity. In addition, factors released from the cells by the action of 1α,25(OH)₂D₃ on the plasma membrane also modulate PKC activity in the extracellular organelle.

EXPERIMENTAL PROCEDURES

Experimental Design—We used two experimental models to examine the regulation of matrix vesicle PKC by 1α,25(OH)₂D₃. In the first set of experiments, we tested the hypothesis that 1α,25(OH)₂D₃ regulates the differential distribution of PKC isoforms during matrix vesicle biogenesis. Rat costochondral growth zone cartilage cells were treated with 1α,25(OH)₂D₃ for up to 24 h. Matrix vesicles and plasma membranes were then isolated from the cultures. The 1α,25(OH)₂D₃-dependent isoform in each membrane fraction was determined using isoform-specific antibodies, comparing the effect at 90 min to the effect at 24 h. We also examined the regulation of matrix vesicle production by 1α,25(OH)₂D₃ using monensin to block protein transport through the Golgi. While it is known that the 1,25-mVDR mediates the rapid increase in PKC at 9 min (16), it is not known if the downstream genomic regulation of matrix vesicle PKC is also regulated via the 1,25-mVDR or any of the signaling pathways. The role of the 1,25-mVDR in the process was assessed using Ab99. We also examined whether the effect of 1α,25(OH)₂D₃ on matrix vesicle PKC at 24 h is mediated by PLC, which was previously shown to mediate the 1,25-mVDR-dependent rapid increase in PKC activity in growth zone cells. For these experiments, cells were treated with 1α,25(OH)₂D₃ in the presence of the phosphatidylinositol-specific (PI-PLC) inhibitor U73122.

The second model used matrix vesicles isolated from cultures not previously treated with 1α,25(OH)₂D₃ to examine the mechanism of the direct effect of the secosteroid. Matrix vesicles were incubated with 1α,25(OH)₂D₃ ± inhibitors of signal transduction pathways shown previously to modulate the activation of PKCα in a number of experimental systems. For these experiments, membrane fractions were incubated with the following: U73122 to inhibit PLC activity; cholera toxin, pertussis toxin, and GDPβS to inhibit G-proteins; and wortmannin to inhibit phosphatase D (PLD). In addition, we examined the regulation of matrix vesicle PKC by agents shown previously to modulate PKCζ activity in growth zone cells. Matrix vesicles were treated directly with arachidonic acid, which is the product of PLA₂ action, the arachidonic acid precursor, linolenic acid, and the arachidonic acid metabolite PGE₂ as well as with diacylglycerol, the product of PLC action. The role of the 1,25-mVDR in the response of matrix vesicle PKC to 1α,25(OH)₂D₃ was assessed using Ab99. Specificity of the response was established using 1×,25(OH)₂D₃ and 24R,25(OH)₂D₃. The role of annexin II was assessed using antibodies to the C-terminal and N-terminal regions of the protein.

Because matrix vesicles do not contain DNA or RNA, any response to the addition of 1α,25(OH)₂D₃ by naive membranes would be prior to genomic mechanisms. This would not rule out a role for the 1,25-mVDR, however. Accordingly, we examined matrix vesicles for the presence of 1α,25(OH)₂D₃-dependent PKC activity via autoradiography using antibodies to the C-terminal and N-terminal regions of the protein. Reagents—Monensin and PGE₂ were purchased from Sigma. The following chemicals were purchased from Calbiochem (San Diego, CA): 1,2-diacyteng-en-glycerol (DOG), arachidonic acid, linoleic acid, pertussis toxin (G inhibitor), cholera toxin (G inhibitor), GDPβS (general G-protein inhibitor), and wortmannin (PLD inhibitor). 1α,25(OH)₂D₃ and 24R,25(OH)₂D₃ were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). Recombinant nuclear vitamin D receptor (1,25-mVDR) was obtained from Affinity BioReagents, Inc., Golden, CO. Rabbit polyclonal anti-1,25-nVDR, and alkaline phosphatase-conjugated goat anti-rabbit antibodies, as well as polyclonal rabbit antibodies specific for the a, b, d, e, and ζ isoforms of PKC, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Nonspecific rabbit IgG1 served as a control.
was obtained from Sigma. Mouse monoclonal antibody to the C terminus of annexin II was obtained from Transduction Laboratories (Lexington, KY), and rabbit polyclonal antibody to the N terminus of annexin II was obtained from Santa Cruz Biotechnology. PKC assay reagents and Dulbecco's modified Eagle's medium were obtained from Life Technologies, Inc. (Gaithersburg, MD). The protein content of each sample was determined using the bicinchoninic acid protein assay reagent (32) obtained from Pierce. 1α,25(OH)2D3 was a generous gift from Dr. Anthony Norman, University of California, Riverside, CA.

Chondrocyte Cultures—The rat costochondral chondrocyte culture system used in this study has been described in detail previously (20). Cells from the growth zone (prehypertrophic and upper hypertrophic cell zones) of costochondral cartilage from 125-g male Sprague-Dawley rats (Harlan, Indianapolis, IN) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, vitamin C, and antibiotics. Fourth passage cells were used for all experiments. The characteristics of these cells have been described in a number of publications and have been reviewed (14, 22).

Membrane Isolation—Matrix vesicles were prepared by differential centrifugation of trypsin digests of the extracellular matrix as previously described (33). In addition, plasma membranes were prepared by differential and sucrose gradient centrifugation of cells isolated from the same cultures for comparison.

Protein Kinase C—PKC activity was determined using previously described methods (19, 23). To determine total PKC specific activity in each culture, cell layer lysates were used. To determine PKC specific activity of isolated matrix vesicles or plasma membranes, 10 μg of membrane protein were diluted to a final volume of 35 μl and assayed as described for the cell layer lysates. For experiments examining the direct effect of hormones and inhibitors on matrix vesicles and plasma membranes, the protein concentration was adjusted with 0.9% NaCl such that 10 μg of membrane protein were incubated with the vitamin D3 metabolites in a final volume of 50 μl. Following incubation for the times shown below, 35 μl was removed and assayed using the same conditions as for cell layer lysates.

To test the hypothesis that 1α,25(OH)2D3 modulates matrix vesicle PKC activity through production of new matrix vesicles and incorporation of PKC, confluent fourth passage cultures of growth zone chondrocytes were incubated with 10−8 or 10−7 M 1α,25(OH)2D3 for 0.2, 1.5, 12, and 24 h. At each time, plasma membranes and matrix vesicles were isolated from the cultures, and PKC specific activity was assayed.

![Fig. 1](image-url) PKC specific activity of matrix vesicles (A) and plasma membranes (B) isolated from growth zone chondrocyte cultures that were treated with 1α,25(OH)2D3 for varying periods of time. All values are the means ± S.E. for membranes from six independent cultures. Data are from one of two separate experiments, both with comparable results. *, p < 0.05, versus control; †, p < 0.05, 10−8 M versus 10−7 M 1α,25(OH)2D3.

![Fig. 2](image-url) Effect of PKC isoform-specific antibodies on PKC specific activity in matrix vesicles isolated from growth zone chondrocyte cultures that were treated with 1α,25(OH)2D3 for 90 min (A) or 24 h (B). Values are the means ± S.E. for matrix vesicle membranes isolated from six independent cultures. Data are from one of two separate experiments, both with comparable results. *, p < 0.05, versus control or preimmune IgG; †, p < 0.05, control versus 1α,25(OH)2D3.
Two separate experiments, both with comparable results. Membranes isolated from six independent cultures. Data are from one of two separate experiments, both with comparable results.

To determine whether the organelle-specific effect of 1α,25(OH)2D3 is due to a change in the differential distribution of PKC isoforms during matrix vesicle biogenesis, isofrom-specific antibodies were used. Growth zone chondrocytes were treated with 10−8 M 1α,25(OH)2D3 for 90 min or 24 h, and matrix vesicle and plasma membranes were isolated. Membranes were depleted of individual isoforms by immunoprecipitation, and the supernatant was assayed for remaining PKC activity (19). Membrane preparations (10 μg of protein/sample) were incubated on ice for 1 h with 6 μl of a 1:10 dilution of nonspecific rabbit IgG1 or isofrom-specific anti-PKC rabbit IgG1 in 0.9% saline, resulting in a final antibody dilution of 1:500. Protein G-agarose (10 μl) (Oncogene Science, Inc., Uniondale, NY) was added for 4 h to clear the samples of immunoreactive PKC isoforms and any remaining unbound antibody. Following precipitation of this material, 35 μl of the supernatant was assayed for PKC activity.

To determine whether protein transport through the Golgi apparatus is necessary for the PKC activity in matrix vesicles, growth zone chondrocyte cultures were treated with 1, 10, or 100 nM 1α,25(OH)2D3. To determine whether the direct effect of 1α,25(OH)2D3 on isolated plasma membranes and matrix vesicles (16). Ab99 also blocks many of the physiological responses of growth zone cells to 1α,25(OH)2D3 (17).

Specificity of the effect was shown using the stereoisomer of 1α,25(OH)2D3, 1β,25(OH)2D3 (provided as a generous gift by Dr. Anthony Norman, University of California, Riverside, CA). Although 1β,25(OH)2D3 blocks 1α,25(OH)2D3-dependent transcalcification in chick intestine (37), it does not affect the rapid increase in PKC due to 1α,25(OH)2D3 in rat growth zone chondrocyte cultures (18). Matrix vesicles were isolated from growth zone chondrocyte cultures that had been treated for 24 h with 10−8 M 1α,25(OH)2D3, 1β,25(OH)2D3, and PKC activity was measured. Specificity was also examined using 24R,25(OH)2D3, a metabolite of vitamin D that does not elicit a rapid increase in PKC in growth zone chondrocyte cultures (23), nor does it affect PKC when incubated with matrix vesicles or plasma membranes isolated from growth zone chondrocyte cultures (19). For these studies, growth zone chondrocytes were treated with 10−7 M 24R,25(OH)2D3 or Ab99 for 24 h. Matrix vesicles were isolated, and PKC activity was determined.

To determine whether signaling pathways that mediate the rapid action of 1α,25(OH)2D3 on PKC in growth zone chondrocytes also mediate the downstream increase in matrix vesicle PKC, we examined the role of PI-PLC. Cultures were treated with 10−8 M 1α,25(OH)2D3, the inhibitor U73122 (0, 0.1, 1, or 10 μM). Matrix vesicles were isolated, and PKC activity was determined.

**Fig. 3. Effect of PKC isofrom-specific antibodies on PKC specific activity in plasma membranes isolated from growth zone chondrocyte cultures that were treated with 1α,25(OH)2D3 for 90 min (A) or 24 h (B). Values are the means ± S.E. for plasma membranes isolated from six independent cultures. Data are from one of two separate experiments, both with comparable results. #, p < 0.05, versus control or preimmune IgG; *, p < 0.05, control versus 1α,25(OH)2D3.**
$^{1\alpha,25}(OH)_2D_3$ Elicits G-protein-dependent Effects on Matrix Vesicles

**Stereospecificity of the Vitamin D$_3$ Effect**

Matrix Vesicle PKC Activity (24 Hours)

![Stereospecificity Graph](Image)

$1\alpha,25(OH)_2D_3$ ± 0.1, 1, or 10 $\mu$m wortmannin, a specific inhibitor of PLD activity (40, 41). Wortmannin at higher concentrations can also inhibit PI 3-kinase (42); however, PI 3-kinase is not affected at the concentrations used in the present study (43).

To determine whether $1\alpha,25(OH)_2D_3$ exerts its direct effects on matrix vesicle PKC through a G-protein-dependent mechanism, matrix vesicles were incubated for 9 min with $10^{-8}$ to $10^{-6}$ $M$ $1\alpha,25(OH)_2D_3$ in the presence of the general G-protein inhibitor GDPBS (1, 10, or 100 $\mu$m), cholera toxin to inhibit $G_s$ (1, 10, or 100 $\mu$m), or pertussis toxin to inhibit $G_i$ (1, 10, or 100 $\mu$m).

The rapid effect of $1\alpha,25(OH)_2D_3$ on PKC in growth zone chondrocytes is mediated by the action of arachidonic acid (44). In addition, linolenic acid, which is the precursor of arachidonic acid, also exerts a stimulatory effect on PKC activity in the cells, as does the arachidonic acid metabolite PGE$_2$ (45). To determine whether one or more of these lipid mediators exerts an effect on matrix vesicle PKC, matrix vesicles were incubated with 1, 10, or 100 $\mu$m arachidonic acid, 1, 10, or 100 $\mu$m linolenic acid, or 0.015, 0.06, or 0.24 $ng/ml$ PGE$_2$ in the presence and absence of $10^{-8}$ to $10^{-6}$ $M$ $1\alpha,25(OH)_2D_3$. The anti-PKC antibody was the $1\alpha,25(OH)_2D_3$-sensitive isoform.

Regardless of the time point examined, the predominant PKC isoform present in plasma membranes was PKC$_{z}$. In control cultures at 90 min, anti-PKC$_{z}$ antibody reduced activity by $\approx 80\%$, whereas none of the other isoform-specific antibodies reduced activity (Fig. 2A). In cultures treated with $1\alpha,25(OH)_2D_3$ for 90 min, the anti-PKC$_{z}$ antibody further reduced PKC specific activity to less than 10% of levels observed in cultures treated with IgG1, indicating that PKC$_{z}$ was the $1\alpha,25(OH)_2D_3$-sensitive isoform. At 24 h, the $1\alpha,25(OH)_2D_3$-dependent increase in PKC activity was also due to PKC$_{z}$ (Fig. 2B). The anti-PKC$_{z}$ antibody reduced $1\alpha,25(OH)_2D_3$-stimulated PKC activity to below baseline levels, although levels still remained higher than in control cultures following precipitation of PKC$_{z}$ with the isoform-specific antibody.

To determine whether the 1,25-vD3 affects plasma membrane PKC only at the early time points, causing a concentration-dependent increase in enzyme activity within 0.2 h (Fig. 1B). Activity remained elevated at 1.5 h in cultures treated with $10^{-8}$ to $10^{-6}$ $M$ $1\alpha,25(OH)_2D_3$, but by 12 h, specific activity had returned to control levels.

The predominant PKC isoform present in matrix vesicles was PKC$_{x}$. In control cultures at 90 min, anti-PKC$_{x}$ antibody reduced activity by $\approx 80\%$, whereas none of the other isoforms reduced activity (Fig. 2A). In cultures treated with $1\alpha,25(OH)_2D_3$ for 90 min, the anti-PKC$_{x}$ antibody further reduced PKC specific activity to less than 10% of levels observed in cultures treated with IgG1, indicating that PKC$_{x}$ was the $1\alpha,25(OH)_2D_3$-sensitive isoform. At 24 h, the $1\alpha,25(OH)_2D_3$-dependent increase in PKC activity was also due to PKC$_{x}$ (Fig. 2B). The anti-PKC$_{x}$ antibody reduced $1\alpha,25(OH)_2D_3$-stimulated PKC activity to below baseline levels, although levels still remained higher than in control cultures following precipitation of PKC$_{x}$ with the isoform-specific antibody.

**RESULTS**

$1\alpha,25(OH)_2D_3$ regulated matrix vesicle PKC in growth zone chondrocyte cultures in a dose- and time-dependent manner. At 0.2 and 1.5 h, $1\alpha,25(OH)_2D_3$ caused a dose-dependent decrease in matrix vesicle PKC specific activity (Fig. 1A). However, at 12 and 24 h, $1\alpha,25(OH)_2D_3$ dose dependently increased PKC activity in these organelles by 100% over control levels. In contrast, $1\alpha,25(OH)_2D_3$ affected plasma membrane PKC only at the early time points, causing a concentration-dependent increase in enzyme activity within 0.2 h (Fig. 1B). Activity remained elevated at 1.5 h in cultures treated with $10^{-8}$ to $10^{-6}$ $M$ $1\alpha,25(OH)_2D_3$, but by 12 h, specific activity had returned to control levels.

For each experiment, each data point represents the means ± S.E. for six individual cultures. Each experiment was repeated two or more times to ensure the validity of the data. The data presented are from a single representative experiment. Significance between groups was determined by analysis of variance and post hoc testing performed using Bonferroni’s modification of Student’s t test for multiple comparisons. p values less than 0.05 were considered significant.

**Statistical Management of Data** — For each experiment, each data point represents the means ± S.E. for six individual cultures. Each experiment was repeated two or more times to ensure the validity of the data. The data presented are from a single representative experiment. Significance between groups was determined by analysis of variance and post hoc testing performed using Bonferroni’s modification of Student’s t test for multiple comparisons. p values less than 0.05 were considered significant.

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Regardless of the time point examined, the predominant PKC isoform present in plasma membranes was PKC$_{x}$ based on the reduction in enzyme activity following incubation with the anti-PKC$_{x}$ antibody (Fig. 3). None of the other isoform-specific antibodies used caused a decrease in control cells. PKC$_{x}$ was also the isoform sensitive to $1\alpha,25(OH)_2D_3$, based on the 70% reduction in total PKC specific activity in plasma membranes isolated from growth zone cells treated with the secosteroid for 90 min. However, the anti-PKC$_{x}$ antibodies did not completely reduce PKC activity to levels seen in control cells.

Incorporation of PKC activity in matrix vesicles was dependent upon protein transport through the Golgi. Treatment of the cultures for 24 h with monensin resulted in a dose-dependent decrease in matrix vesicle PKC specific activity (Fig. 4). In contrast, PKC activity of the plasma membranes was unaffected by treatment of the cultures with monensin.

The stimulatory effect of $1\alpha,25(OH)_2D_3$ on matrix vesicle PKC was receptor-mediated. Not only was it stereospecific, but it
1α,25(OH)2D3 Elicits G-protein-dependent Effects on Matrix Vesicles

**Fig. 6. Role of membrane-mediated signaling mechanisms in the 1α,25(OH)2D3-dependent decrease in matrix vesicle PKC.**

**A.** Role of 1,25-mVDR. Matrix vesicles were treated for 9 min with 1α,25(OH)2D3 in the presence/absence of Ab99. **B.** Role of PLC. Matrix vesicles were treated with PI-PLC inhibitor (U73122; 10 μM), 1α,25(OH)2D3 (10−8 M), or the two together for 3, 9, 30, or 90 min. **C.** Role of G-protein. Matrix vesicles were treated for 9 min with GDPβS in the presence/absence of 10−8 M 1α,25(OH)2D3. Values are the means ± S.E. for six separate membrane preparations, each from an individual culture. Data are from one of two independent experiments, both with comparable results. *p < 0.05, versus untreated control (A); versus 3 min (B); or versus no G-protein inhibitor (C); #, p < 0.05, Ab99 versus control (A); versus control or U73122 for a particular time (B); or versus 1α,25(OH)2D3 (C).

was metabolite-specific. Neither 1α,25(OH)2D3 nor 24R,25(OH)2D3 elicited the response (Fig. 5A). Ab99 blocked the increase in matrix vesicle PKC due to 1α,25(OH)2D3 but had no effect on PKC in control cultures or in cultures treated with 1α,25(OH)2D3 or 24R,25(OH)2D3, indicating that the 1,25-mVDR was involved.

Part of the long-term effect of 1α,25(OH)2D3 on matrix vesicle PKC involves a PI-PLC-dependent mechanism. U73122 caused a dose-dependent decrease in matrix vesicle PKC activity (Fig. 5B). At the highest concentration of the PI-PLC inhibitor, there was a 30% reduction in the 1α,25(OH)2D3-stimulated PKC activity.

The direct effect of 1α,25(OH)2D3 on PKC in isolated matrix vesicles was also mediated by the 1,25-mVDR. When matrix vesicles were incubated with 1α,25(OH)2D3, Ab99 prevented the dose-dependent decrease in PKC activity, even at the highest concentration of the secosteroid (Fig. 6A). Ab99 did not alter the 24R,25(OH)2D3-dependent decrease in PKC observed in matrix vesicles isolated from resting zone cell cultures (data not shown), indicating that the effect of the antibody was specific to the 1,25-mVDR and not due to a nonspecific interaction with the matrix vesicle or PKC. Moreover, Ab99 caused a dose-dependent reversal of the direct effect of 1α,25(OH)2D3 on matrix vesicle PKC, but increasing concentrations of Ab99 did not affect PKC activity of control matrix vesicles (Table I). Furthermore, the direct effect of 1α,25(OH)2D3 on matrix vesicle PKC was not via annexin II. Antibodies to either the C or N terminus of the protein had no effect on 1α,25(OH)2D3-dependent PKC inhibition, nor did they affect PKC activity of control matrix vesicles (Table I).

PI-PLC was not involved in the 1α,25(OH)2D3-dependent decrease in matrix vesicle PKC since U73122 did not alter the inhibitory effect of the secosteroid (Fig. 6B). In addition, PKC in control matrix vesicles was not sensitive to PLC inhibition by U73122. Moreover, matrix vesicle PKC activity was insensitive to DOG, whether the organelles were treated with 1α,25(OH)2D3 or not (data not shown), indicating that diacylglycerol was not involved. The direct effect of 1α,25(OH)2D3 on matrix vesicle PKC was also mediated by the 1,25-mVDR but increasing concentrations of Ab99 did not affect PKC activity of control matrix vesicles (Table I).

1α,25(OH)2D3 mediates its direct effect on matrix vesicle PKC via a G-protein-dependent mechanism. Treatment of matrix vesicles with the general G-protein inhibitor GDPβS resulted in an increase in PKC activity of control matrix vesicles and a reversal of the 1α,25(OH)2D3-dependent decrease in PKC (Fig. 6C). The G-protein responsible is neither Gi nor Gs, since cholera toxin and pertussis toxin had no effect on PKC activity of control matrix vesicles or 1α,25(OH)2D3-treated matrix vesicles (data not shown).

Matrix vesicle PKC was directly regulated by linoleic acid. Linoleic acid caused a rapid, dose-dependent increase in matrix vesicle PKC, whereas this fatty acid did not alter enzyme activity of isolated plasma membranes (Fig. 7A). The increase in matrix vesicle PKC was time-dependent (Fig. 7B). By 90 min, the direct effect on matrix vesicles was reduced by 30%. Even at 270 min, however, linoleic still exerted a stimulatory effect on matrix vesicle PKC compared with control matrix vesicles.

Arachidonic acid also directly regulated matrix vesicle PKC activity. In both control matrix vesicles and in matrix vesicles treated directly with 1α,25(OH)2D3, arachidonic acid caused a rapid increase in PKC (Fig. 6A). The effect of arachidonic acid
was time-dependent (Fig. 8B). Maximal increases in PKC were found in matrix vesicles incubated with arachidonic acid for 90 min. Moreover, only at this time point was the 1α,25(OH)₂D₃-dependent decrease in PKC restored to control levels by arachidonic acid. In contrast to the stimulatory effects of linoleic acid and arachidonic acid on PKC activity, the arachidonic acid metabolite PGE₂ had no effect on enzyme activity in control matrix vesicles (data not shown). Similarly, PGE₂ did not alter the inhibitory effect of 1α,25(OH)₂D₃.

The direct effect of 1α,25(OH)₂D₃ on matrix vesicle PKC was not due to the nuclear VDR. Matrix vesicles and plasma membranes isolated from growth zone chondrocyte cultures contained no detectable immunoreactive 1,25-nVDR (Fig. 9). The cytosol/nuclear fraction remaining after matrix vesicle and plasma membrane isolation did contain 1,25-nVDR immunoreactivity. Immunoreactive bands were seen at 63 and 297 kDa in the growth zone chondrocyte cytosol/nuclear fraction as compared with recombinant 1,25-nVDR, which was detected as a single band with a molecular mass of 63 kDa.

**DISCUSSION**

The results of this study indicate that 1α,25(OH)₂D₃ exerts its effects on matrix vesicle PKC activity through both direct and indirect mechanisms. The initial effect of the secosteroid is to cause the rapid decrease in existing matrix vesicle PKC activity by down-regulating PKCζ. It is likely that this is due to direct action of 1α,25(OH)₂D₃ on the matrix vesicle membrane. At later time points, however, 1α,25(OH)₂D₃ causes an increase in matrix vesicle PKCζ activity, suggesting that PKC is regulated at the time of matrix vesicle formation through genomic mechanisms.

The fact that monensin-treated cultures exhibit reduced PKC activity in their matrix vesicles, but not in their plasma membranes, indicates that less PKC is transported and packaged within matrix vesicles. This observation suggests that matrix vesicles are formed within the Golgi, independently of the plasma membrane. Studies using somatic cell hybrids to examine the role of alkaline phosphatase in osteoblasts demonstrated that alkaline phosphatase is packaged in matrix vesicles even when expression of the plasma membrane enzyme is extinguished (48), supporting this hypothesis.

At least part of the downstream effect of 1α,25(OH)₂D₃ on matrix vesicle PKC is mediated by the 1,25-mVDR. Treatment of the cultures for 24 h with Ab99 blocked the 1α,25(OH)₂D₃-dependent increase in matrix vesicle PKC activity. The failure of either 1β,25(OH)₂D₃ or 24R,25(OH)₂D₃ to stimulate matrix vesicle PKC or for Ab99 to inhibit matrix vesicle PKC in cells treated with 1β,25(OH)₂D₃ or 24R,25(OH)₂D₃ demonstrates the specificity of the 1,25-mVDR-activated response. It is possible that the effect of Ab99 was directly on matrix vesicles already present in the extracellular matrix. However, it is more likely that it blocked the plasma membrane 1,25-mVDR since it inhibited the 1α,25(OH)₂D₃-dependent increase in enzyme activity, which is cell-mediated.

Further evidence that the 1,25-mVDR is involved is the decrease in 1α,25(OH)₂D₃-dependent matrix vesicle PKC noted in growth zone cells in the presence of U73122. This PI-PLC inhibitor also blocks the rapid effects of 1α,25(OH)₂D₃ on PKC activity that are mediated by the 1,25-mVDR in growth zone cells (23). However, U73122 did not affect existing PKC activity in matrix vesicles incubated directly with the secosteroid, indicating that the changes in matrix vesicle PKC observed in cultures treated with 1α,25(OH)₂D₃ for 24 h were not due to direct effects of the secosteroid on existing matrix vesicles. Instead, they were due to indirect effects involving 1,25-mVDR-dependent PKC synthesis and packaging in matrix vesicles through PLC-dependent signaling pathways, resulting in new PKC expression. Because the primary isoform active in matrix vesicles at 90 min and 24 h is PKCζ, the data suggest that PI-PLC activity is necessary for the production of PKCζ-enriched matrix vesicles rather than having a direct effect on existing matrix vesicle PKCζ. This is consistent with the matrix vesicle PKC being PKCζ because it is diacylglycerol-independent and would not require the production of diacylglycerol by PLC (25). Similarly, DOG, a potent PKC-activating form of diacylglycerol, was shown in this study to be ineffective at activating matrix vesicle PKC activity when matrix vesicles were treated directly with the compound.

It is likely that MAP kinase was involved in the 1α,25(OH)₂D₃-dependent increase in matrix vesicle PKC activity. 1α,25(OH)₂D₃ has been shown to mediate its effects on enteroctyes (49) and osteoblasts (50) via MAP kinase signaling pathways, and the rapid increase in cellular PKC in response to 1α,25(OH)₂D₃ can result in increased MAP kinase activity (51). Although 1α,25(OH)₂D₃ mediates many of its physiological effects on growth zone chondrocytes through mechanisms that include the 1,25-mVDR-dependent signaling pathways (17), it is important to note that traditional 1,25-nVDR mechanisms are also likely to be involved in the packaging of matrix vesicle PKC in the present study. 1,25-nVRs are present in the cells, as demonstrated by the presence of immunoreactive receptors in all matrix vesicle preparations.
Western blots of the cytosol/nuclear fraction of the growth zone chondrocytes. The dependence of the downstream increase in matrix vesicle PKC activity on the 1,25-mVDR suggests that the action of 1α,25(OH)2D3 on these two receptors is interrelated. 1α,25(OH)2D3 also exerts its direct effects on matrix vesicle PKC through receptor-mediated mechanisms. The 1,25-mVDR is involved since Ab99 reversed the 1α,25(OH)2D3-dependent decrease in PKC activity. The 1,25-nVDR does not appear to play a role in the direct action of 1α,25(OH)2D3 on matrix vesicles, however. We failed to identify the 1,25-nVDR on Western blots of either the matrix vesicles or plasma membranes isolated from growth zone chondrocyte cultures, although it was present in the cytosol/nuclear fraction of these cells.

Annexin II has also been reported to be a membrane receptor for 1α,25(OH)2D3 (46), and annexin II is present in matrix vesicles produced by chick growth plate chondrocytes (52) and osteoblast-like cells (53). It is unlikely that annexin II is responsible for the 1α,25(OH)2D3-dependent effects on matrix vesicles, however. Antibodies to annexin II failed to block the direct inhibition of matrix vesicle PKC by 1α,25(OH)2D3. This confirms our earlier observation using osteoblast cell lines (53). Ab99 did not cross react with annexin II in Western blots of matrix vesicles produced by osteoblast cultures, nor did antibodies to annexin II block the 1α,25(OH)2D3-dependent increase in PKC in these cells.

The mechanism by which 1α,25(OH)2D3 regulates matrix vesicle PKC are different from those that mediate the regulation of cellular PKC. PI-PLC is not involved, nor is diacylglycerol. PLD is also not involved, based on the lack of an effect of wortmannin either on PKC activity of control matrix vesicles or on PKC activity of 1α,25(OH)2D3-treated matrix vesicles. In contrast, both cellular PKC and matrix vesicle PKC are regulated by G-protein-dependent mechanisms. As noted for intact

***Fig. 7. Direct effect of linolenic acid on PKC activity in isolated matrix vesicles and plasma membranes.*** Matrix vesicles (MV) and plasma membranes (PM) from growth zone chondrocyte cultures were treated with varying doses of linolenic acid for 9 min (A). Alternatively, matrix vesicles were treated with 10 μM linolenic acid for 9, 90, or 270 min (B). Values are the means ± S.E. for membranes isolated from six independent cultures. Data are from one of two independent experiments, both with comparable results. *, p < 0.05, versus no linolenic acid (A) or 9 min (B); #, p < 0.05, MV versus PM (A) or control versus linolenic acid (B); ●, p < 0.05, versus 1 μM linolenic acid (A).

***Fig. 8. Direct effect of arachidonic acid on PKC activity in matrix vesicles.*** Matrix vesicles were treated with arachidonic acid for 9 min in the presence/absence of 10−8 M 1α,25(OH)2D3 (1,25) (A). Alternatively, matrix vesicles were treated with arachidonic acid (100 μM), 1α,25(OH)2D3 (10−8 M), or the two together for 9, 90, or 270 min (B). Values are the means ± S.E. for membranes isolated from six independent cultures. Data are from one of two independent experiments, both with comparable results. *, p < 0.05, versus untreated control (A) or versus 9 min (B); *, p < 0.05, 1α,25(OH)2D3 versus −1α,25(OH)2D3 (A) or versus untreated control for a particular treatment time (B).
1α,25(OH)₂D₃ Elicits G-protein-dependent Effects on Matrix Vesicles

cells (38), neither Gₛ nor Gₛ is involved, either in maintenance of PKC activity in control matrix vesicles or in the decrease noted in matrix vesicles treated with 1α,25(OH)₂D₃. However, inhibition of G-protein activity with GDP/BS blocks the effects of 1α,25(OH)₂D₃. Thus, Gₛ is the likely candidate because it is not sensitive to pertussis toxin or cholera toxin (54). This is the case for intact cells as well (21).

1α,25(OH)₂D₃ causes an increase in matrix vesicle phospholipase A₂ activity (28), suggesting that release of arachidonic acid might be involved in the mechanism of its action on matrix vesicle PKC. However, the direct effect of 1α,25(OH)₂D₃ is a decrease in PKC activity, and exogenous arachidonic acid elicits a rapid increase. Others have noted that arachidonic acid can stimulate PKC directly, particularly the PKCs isoform (55), explaining in part how this fatty acid can increase PKC activity in growth zone chondrocyte cultures (44). It is likely that arachidonic acid did not cause an increase in PKC activity of the isolated matrix vesicles because PKCζ is relatively insensitive to the fatty acid (56). It is more likely that arachidonic acid is exerting its stimulatory effect on the low levels of PKCζ present in the organelles (19).

One way the chondrocytes might be able to activate matrix vesicle PKC activity after the matrix vesicles are formed and released into the extracellular matrix would be to secrete a regulatory factor that could act remotely on the organelles. We have shown that 1,25(OH)₂D₃ is produced by the cells, suggesting that it could serve this purpose. 1α,25(OH)₂D₃ can also act back on the cell or matrix vesicle to stimulate the rapid release of arachidonic acid. We have previously reported that 1α,25(OH)₂D₃ stimulates arachidonic acid turnover in growth zone chondrocytes (57) and increases PLA₂ activity in the cells (5). Thus, a regulatory feedback loop can be established. 1α,25(OH)₂D₃ decreases matrix vesicle PKCζ and arachidonic acid released by the action of 1α,25(OH)₂D₃ increases PKCζ activity.

If anything, the stimulatory effect of linoleic acid on matrix vesicle PKC activity is even more robust than the effect of its metabolite, arachidonic acid. Moreover, the effect of linoleic acid is specific to matrix vesicles; when plasma membranes were incubated directly with this fatty acid, no change in PKC activity was observed. Linoleic acid has been shown to be a potent activator of PKCζ (56). The significance of this is unclear at this time.

In contrast to the stimulatory effects of arachidonic acid and its biosynthetic precursor linoleic acid, the arachidonic acid metabolite PGE₂ had no effect on matrix vesicle PKC. In intact cultures, PGE₂ mediates the stimulatory effects of 1α,25(OH)₂D₃ on PKCζ through its action on EP₁ receptors (18). Whether EP₁ is also present in matrix vesicles is not yet known, nor is it known if matrix vesicles contain cyclooxygenase activity.

Fig. 10 illustrates our current model of 1α,25(OH)₂D₃ regulation of matrix vesicle PKC activity. 1α,25(OH)₂D₃ binds to the 1,25-mVDR on the plasma membrane, triggering the production of matrix vesicles containing increased PKCζ activity through genomic mechanisms mediated by PKC and MAP kinase as well as by the 1,25-nVDR. PLA₂ activity is also increased, generating arachidonic acid, which acts on matrix vesicles in the extracellular matrix, increasing PKC activity. In addition, 1α,25(OH)₂D₃ acts directly on matrix vesicles, activating the 1,25-mVDR and causing a rapid decrease in PKCζ activity through a mechanism involving Gₛ. The combination of an increase in PKCζ and a decrease in PKCζ modulates activity of matrix metalloproteinases present in the matrix vesicles (58), leading to growth factor activation, matrix remodeling, maturation, calcification, and endochondral ossification (8, 24, 59).

In summary, these results show that the 1,25-mVDR is present in matrix vesicles from growth zone chondrocytes, whereas the 1,25-nVDR is not. 1α,25(OH)₂D₃ can regulate matrix vesicle PKC activity both directly and indirectly. Both processes involve the 1,25-mVDR, but use different pathways to elicit their effects. 1α,25(OH)₂D₃-dependent activation of the 1,25-

Fig. 9. Western blot analysis of nuclear vitamin D receptor (1,25-nVDR) in growth zone chondrocyte (GC) membrane fractions. Matrix vesicles (MV), plasma membranes (PM), and cytosol/nuclear fractions (Cyto+Nuc) were analyzed for the presence of the 1,25-nVDR. The 63-kDa band present in the cytosol/nuclear fraction was verified to be nVDR by comparison to recombinant human 1,25-nVDR. The 63-kDa band present in the cytosol/nuclear fraction (19).

Fig. 10. Proposed model of matrix vesicle regulation by 1α,25(OH)₂D₃. 1α,25(OH)₂D₃ activates the 1,25-mVDR, initiating a rapid increase in PKCζ activity via PLC-dependent production of DAG and translocation of PKCζ to the membrane. The ensuing phosphorylation cascade and subsequent binding of 1α,25(OH)₂D₃ to the 1,25-nVDR results in new PKCζ expression and packaging into matrix vesicles. Matrix vesicles continue to mature in the matrix, eventually supporting the formation of calcium phosphate crystals on the inner leaflet of the membrane, eventual loss of membrane integrity, and exposure of matrix vesicle contents to the extravesicular environment. Arachidonic acid released by cellular phospholipase A₂ stimulates matrix vesicle PKC, while 1α,25(OH)₂D₃ secreted by the cell inhibits matrix vesicle PKCζ. MV = matrix vesicles; AA = arachidonic acid.
mVDR in growth zone cells results in an increase in production of new matrix vesicles that are enriched in PKCζ through a mechanism involving PI-PLC. In matrix vesicles, 1α,25(OH)₂D₃ mediates its 1,25-mVDR-dependent effects on PKCζ through a mechanism that involves G, but not PI-PLC or PLD. Matrix vesicle PKC is also stimulated by arachidonic acid and linolenic acid, suggesting additional pathways for modulating the activities of the extracellular organelles. These findings demonstrate that matrix vesicles are unique organelles that can be differentially regulated at the time of their production and at sites distant from the cell surface, either through direct action of 1α,25(OH)₂D₃ or by regulatory factors such as arachidonic acid. This demonstrats the complexity of the role of 1α,25(OH)₂D₃ in chondrocyte biology and indicates that 1α,25(OH)₂D₃ acts both through nuclear and membrane receptors to regulate the biomineralization processes.

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1α,25(OH)₂D₃ Regulates Chondrocyte Matrix Vesicle Protein Kinase C (PKC) Directly via G-protein-dependent Mechanisms and Indirectly via Incorporation of PKC during Matrix Vesicle Biogenesis

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